DECLARATION

I, Michel Schneider, do hereby declare and state that:

1. I am a coinventor of USSN Application 08/456,385.

2. The following comparative experiments have been conducted either by myself or under my direct supervision and control, comparing the properties of air microbubble suspensions in which microbubbles are stabilized with saturated and unsaturated phospholipids.

3. The suspensions of air microbubbles used in the study were prepared as follows:

A liposome solution (50 mg lipids per ml) was prepared in distilled water by the REV method using hydrogenated soy lecithin (NC 95 H, Nattermann Chemie, Köln, W. Germany) and dicetylphosphate in a molar ratio 9/1. The liposome preparation was extruded at 65°C through a 1 µm polycarbonate filter (Nucleopore). Two ml of the liposome preparation were added to 8 ml of 15% maltose solution in distilled water, the resulting solution was frozen at -30°C and then lyophilized under reduced pressure (0.1 Torr) over night. Complete sublimation of the ice was obtained. Thereafter, air pressure was restored in the evacuated container so as to saturate the lyophilized powder with air.

The air saturated dry powder was then dissolved in 10 ml of sterile water under gentle mixing and a microbubble suspension with 2.24×10^8 microbubbles per ml (dynamic viscosity < 20 mPa.s) was obtained. This suspension containing mostly bubbles in the 1-10 µm range was stable for a long period.

The same experiment was repeated under the identical contitions using the same amounts of natural (unsaturated) soy and egg lecithins (obtained from Avanti Polar Lipids, Alabaster, USA). The air saturated dry powders were then dissolved in 10 ml of sterile water under gentle mixing and the microbubble suspensions with microbubble concentrations as above

were obtained. The results reported below are average values obtained from three independent experiments.

Saturated soy lecithin	Unsaturated soy lecithin	Unsaturated egg lecithin
No of microbubbles/ml immediately after preparation		
2.24×10^8	9.0×10^5	1.5×10^6
No of microbubbles/ml 60 minutes after preparation		
9.0×10^7	below 2.0×10^4	below 5.0×10^4

The microbubble characterisations of the suspensions obtained were carried out immediately upon preparation and then 60 minutes after storage at room temperature. Measurements carried out after 120 minutes showed 7.5×10^7 microbubbles per ml for the saturated soy lecithin and unreproducible levels of microbubbles for the unsaturated phospholipids. It is believed that the unreproducibility of the results for the samples with unsaturated phospholipids was due to the very low levels of the microbubbles in the suspensions which were close to the limit of detectablility of the instrument.

4. Characterisation of the microbubbles in the suspensions were carried out using Coulter® Multisizer II instrument connected to a microcomputer for data processing (AccuComp® Coulter Electronics Ltd. London, UK). The Coulter® Multisizer II consists of a sampling standard unit connected to the Multisizer II (an instrument used for setting up menu, data acquisition and visual display of the particle distrubution curve). The sampling unit includes an orifice tube with electrodes on either side of the orifice and a stirrer immersed in a sample beaker. The Coulter® Multisizer II determines the number and size of particles suspended in an elecrolyte solution. The stirrer keeps the particles suspended in the solution during the measurements. A pre-determined volume of the suspension is forced through the orifice tube and the number and the size of the microbubbles is determined as a function of the change of the resistance of the solution between the electrodes.

5. Experiments carried out with unsaturated (soy or egg lecithins) vs. saturated phospholipids have shown quite clearly that unsaturated lecithins are inferior stabilisers of gas microbubbles than the saturated ones. From the results it further follows that unsaturated soy lecithins are less effective as the gas microbubble stabilisers/surfactants than yolk lecithins.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and like so made are punishable by fine or imprisonment, or both under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardise the validity of the application or any patent issuing thereon.

Michel Schneider

Dr. Michel Schneider

Geneva, February 3rd, 1997